Identification of a Genetic Marker to Discriminate Between Old-World and New-World Ciscoes

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Erratum

Hisanaga, T.L., R. Bajno, T.J. Carmichael, and J.D. Reist. 2003. Identification of a genetic marker to discriminate between old-world and new-world Ciscoes. Can. Tech. Rep. Fish. Aquat. Sci. 2485: iv + 20 p.

Page iv: ABSTRACT

Page 1:line 41 should read "Hisanaga *et al.*, 2003"

Page 4: line 41 the acknowledgement should include "A. Kaupinis"

Page 5: line 21 the reference should include

Hisanaga, T.L., R. Bajno, T.J. Carmichael, and J.D. Reist. 2003. Analytical comparison of the genomic mitochondrial DNA of eleven Coregonines. Can. Tech. Rep. Fish. Aquat. Sci. 2486: vi + 39 p.

Page 7: line1 and 25 should read "Barrow Lake"

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by

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ABSTRACT

Hisanaga, T.L., R. Bajno, T.J. Carmichael, and J.D. Reist. 2003. Identification of a genetic marker to discriminate between old-world and new-world Ciscoes. Can. Tech. Rep. Fish. Aquat. Sci. 2485: iv + 20 p.

A portion of the D-loop region (550-600 base pairs) of the mitochondrial DNA was amplified using the polymerase chain reaction and directly sequenced for 15 taxa believed to be representative of the old-world and new-world Coregonines. A number of variable motifs were observed that included substitutions, insertions, deletions, and tandem repeats. Due to the autapomorphic insertions and deletions among the taxa examined, no phylogeny was established. However, a significant large deletion (PyrPurAPyrPurAAA[T or G]TTTATTAATGTATAATAAAAAGTT) occurred among the new-world ciscoes and in *C. autumnalis* (pollan) from Lough Neagh, Ireland. This finding further affirms the phylogenetic relatedness of *C. autumnalis* (pollan) to be more closely associated with the new-world rather than the old-world ciscoes. A second feature of this insertion-deletion is its potential use as a genetic marker to differentiate new-world from old-world ciscoes.

Keywords: D-loop, mitochondrial DNA, phylogenetic relationships, Stenodus, Coregonus

RÉSUMÉ

Hisanaga, T.L., R. Bajno, T.J. Carmichael, and J.D. Reist. 2003. Identification of a genetic marker to discriminate between old-world and new-world Ciscoes. Can. Tech. Rep. Fish. Aquat. Sci. 2485: iv + 20 p.

On a amplifié une partie de la boucle D (550-600 paires de base) de l'ADN mitochondrial en se servant de la réaction en chaîne de la polymérase, puis on l'a séquencée directement chez 15 taxons considérés comme représentants des Corégonidés de l'Europe et du Nouveau Monde. Un certain nombre de motifs variables ont été observés, y compris des substitutions, des insertions, des délétions et des séquences répétées en tandem. À cause des insertions et des délétions autapomorphes chez les taxons examinés, aucune phylogénie n'a été établie. Cependant, une délétion d'envergure importante (PyrPurAPyrPurAAA[T ou G]TTTATTAATGTATAATAAAAAGTT) a été identifiée chez les ciscos du Nouveau Monde et chez *C. autumnalis* (*pollan*) de Lough Neagh, Irlande. Cette découverte étaye davantage le degré de relation phylogénétique de *C. autumnalis* (*pollan*), qui serait plus étroitement apparenté aux ciscos du Nouveau Monde qu'aux ciscos de l'Europe. Une deuxième caractéristique de cette insertion-délétion est qu'elle peut servir de marqueur génétique pour distinguer les ciscos du Nouveau Monde de ceux de l'Europe.

Mots clés : Boucle D, ADN mitochondrial, relations phylogénétiques, Stenodus, Coregonus

INTRODUCTION

At present, Coregonine fishes are divided into three genera: *Prosopium*, *Stenodus*, and *Coregonus* (Nelson, 1994). The genus *Coregonus* is further divided into two subgenera, (*Coregonus*), the true whitefish, characterized by sub-terminal mouths, comparatively fewer gillrakers, and a benthic feeding habit; and (*Leucichthys*), the ciscoes with terminal mouths, a pelagic feeding habit, and a greater number of gillrakers. However, evidence suggests that these sub-genera are not monophyletic (Nelson, 1994).

Furthermore, these taxonomic relationships among the Coregonines are at present unclear. This is due in large part to the meristic and morphometric characteristics used as identifying features for fish. Among the Coregonines, these features are phenotypically plastic and subject to environmental modification (Todd *et al.*, 1980).Therefore, it would be useful to revisit the classifications of Coregonines based upon genetic differences.

Mitochondrial DNA (mtDNA) is one such genetic characteristic that has recently been exploited. It is maternally inherited, lacks recombination, evolves more rapidly than nuclear DNA, and has a rapid sorting time, which make it ideally suited to delineate matriarchal lineages across taxa (Billington and Hebert, 1991). These features also provide the basis for species-specific genetic markers. The D-loop region of the mitochondrial DNA has a left and a right domain, which contain the main regulatory elements, and a central conserved domain (Lee *et al.*, 1995). In general, the right domain has been shown to have evolved rapidly in a species-specific manner, generating heterogeneity in both length and base composition (Lee *et al.*, 1995, Saccone *et al.*, 1991). The right domain is also prone to base pair substitution, the generation of short repeats by replication slippage (Brzuzan, 2000), as well as the insertion and deletion of elements ranging in size from single nucleotides to short stretches (100 base pairs) of DNA (Saccone *et al.*, 1991).

One of the earliest studies focussing on the use of genetic characteristics to determine phylogenetic relationships among the Coregonines involved the electrophoretic comparison of the Irish pollan with other Holarctic Coregoninae (Ferguson *et al.*, 1978). The Irish pollan (*Coregonus autumnalis* pollan) was found to be conspecific with the Arctic species *C. autumnalis* and not with other European (old-world) coregonines. This taxonomic grouping was further supported by direct sequencing of DNA (Reist *et al.*, 1998), allele frequencies (Bodaly *et al.*,1994, 1991), and restriction fragment length polymorphisms (RFLP) (Bernatchez *et al.*, 1991).

Previous work utilizing direct sequencing of mtDNA conducted at the Freshwater Institute Science Laboratory fish genetics laboratory focussed primarily upon a small portion of the entire mitochondrial genome, approximately 400 base pairs of D-loop (Reist *et al.*, 1998). This work demonstrated that variation in the D-loop region differentiated major lineages of Coregonines (e.g., whitefishes versus ciscoes). More recent research examining a longer portion of the D-loop sequence revealed that many of the ciscoes originating in the new-world (e.g., *C. artedi, C. zenithicus*, and *C. hoyi*) appeared to have a deletion of approximately 17 base pairs when compared to old-world ciscoes (*C. sardinella*, and *C. autumnalis baicalensis*) (Hisanaga *et al.* 2003, in press). This deletion was also observed in *C. autumnalis* (pollan) sampled from Lough Neagh in Northern Ireland, leading to a hypothesis that this type of Coregonine may have been responsible for populating the new world.

Based upon the aforementioned features, and the current absence of any known genetic marker to differentiate between old-world and new-world ciscoes, it would appear that the right domain of the D-loop region is the most likely candidate to find such a marker. The purpose of this study was to broaden the fish sampled in terms of nominate taxa, as well as location (Table 1), in an attempt to discover a genetic marker to differentiate old and new-world ciscoes.

MATERIALS AND METHODS

A total of fifteen species (Table 1) were analyzed for a portion of their D-loop sequence. Total DNA (mitochondrial and genomic) was isolated from tissue[1] using DNeasy tissue kits (Qiagen, Mississauga, ON.) as per manufacturer's recommendations, and stored at –20°C. Polymerase chain reaction (PCR) mixtures were prepared containing 0.8 mM forward and reverse primers, 0.1 mM dNTPs (except where otherwise indicated in Appendix 2), 1 X *Taq* polymerase buffer with 1.5 mM $MgCl_2$ (Roche Diagnostics, Laval, QC.), 10-60 ng of DNA, and 0.5 U *Taq* polymerase in a 100 µl reaction volume. Amplification of mtDNA was performed in a Techne Genius thermal cycler using the reverse primer HN20 (5'-GTGTTATGCTTTAGTTAAGC-3') (Bernatchez and Danzmann, 1993) and forward primer Char2 (5'-CAAAACTCCAACTAACACGG-3') (R. Bajno). The thermal cycling parameters for PCR were as follows: initial denaturation at 95 °C for 2 minutes, then thirty cycles of: denaturation for 1 minute at 94 °C, annealing at 55 °C for 45 seconds, extension at 72 °C for 2 minutes, for 30 cycles. Successful amplification of the various mtDNA portions was verified by running 5 µl of the PCR product on a 1.0% agarose gel stained with ethidium bromide (1 mg/ml).

Double stranded DNA products from PCR were purified by filtration through QIAquick columns (Qiagen, Mississauga, ON.) as per manufacturer's recommendations, and stored at -20 °C until future use. The PCR products were then used for direct cycle sequencing with the d-Rhodamine terminator cycle sequencing kit (Applied Biosystems Inc, Foster City, CA) according to the manufacturer's instructions. Sequencing thermal cycling parameters were as follows: initial denaturation at 94 °C for 4 minutes, then 25 cycles of: denaturation for 1 minute at 94 °C, annealing for 35 seconds at 53 °C, extension for 4 minutes at 72 °C. The sequencing primer, DlpPhe (5'-TATGCTTTAGTTAAGGCTACG-3', R. Bajno) was used. The sequencing products were then purified using spin columns (Bio Systems, Gaithersburg, MD.) as per the manufacturer's recommendations.

Sequencing reactions were run and labelled fragments were analyzed on an ABI Prism Model 377 DNA sequencer (Foster City, CA). Sequences were then aligned and edited in MacVector 7.0 (Accelrys Inc., Madison, WI.). Further alignment of the gene-coding and control regions was performed using ClustalW (Thompson *et al.*, 1994).

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[1] Tissues can be muscle, fin clips, adipose fin, or any other tissue removed from either live-released or dead-sampled fish and preserved frozen, in ethanol, or in a NaCl-saturated 20% dimethyl sulphoxide solution at 1:2 (v/v).

RESULTS AND DISCUSSION

ClustalW alignment of the various Coregonines resulted in an alignment in which the conserved blocks were fairly well aligned, however, the beginning of some sequences were not as strongly aligned (data not shown). The final alignment was produced manually based upon the original clustalW alignment (Table 2), and while not definitive, allows for the identification of most of the conserved elements defined in Teleosts (Lee *et al.*, 1995).

An often repeated sequence similar to the light strand-promoter-like sequences (LSPs) (Saccone *et al.*, 1991) was found at the extreme 3' end of the right domain along the light strand in the *C. zenithicus* and *C. artedi* sequenced by Reed *et al.* (1998). The sequencing primer used in this study binds to the opposite strand to that by Reed *et al.* (1998), thus this sequence was also found (Table 2) but reads AAAAGTTPyrAPyrGTAATGTA. This is the reverse and complement to the light strand promoter-like sequence found by Reed *et al.* (1998).

Unfortunately, due to the peculiar evolution of the right domain, the control region for D-loop sequences, cannot be used to determine in a quantitative way the genetic distances between species. However, qualitative relationships are easily discernible from the alignment (Table 2). The new-world ciscoes (*C. artedi, C. zenithicus*, *C. hoyi, C. laurettae*, and *C. autumnalis*) align strongly with one another, with no significant insertions/deletions (in-dels) noted, and very few base substitutions. Also located within this grouping is *C. autumnalis* pollan from Northern Ireland, which is in agreement with the phylogenetic conclusion of other researchers (Bernatchez *et al.*, 1991, Bodaly *et al.*, 1994, 1991, Ferguson *et al.*, 1978, Lockwood *et al.*, 1993, Reist *et al.*, 1998).

The old-world ciscoes and whitefishes examined herein aligned well together, with very close groupings observed between *C. nasus*, *C. autumnalis baicalensis*, and one haplotype of *C. peled* (Table 2). The other haplotype of *C. peled* aligned strongly with both haplotypes of *C. albula* and one of the *C. sardinella* haplotypes. Surprisingly, the whitefish did not align as strongly with one another as they did with other species. *C. clupeaformis* (Mississippi form) aligned well with *C. tugun*, the two being nearly indifferentiable, whereas the Bering form of *C. clupeaformis* showed strongest similarity to the second *C. sardinella* haplotype. Even so, the Bering form of *C. clupeaformis* is still quite distinct. Of the two forms of *C. clupeaformis*, the Bering form appears to be most closely related to the new-world ciscoes (Table 2). This form shares greater identity than the Mississippian form, although the sequence is shorter in length.

There are two major areas of differentiation between old-world and new-world ciscoes as depicted by the alignment profile (Table 2). The sequence TAAGTATAATAAA is found among the new-world members, including the pollan form of *C. autumnalis*, as a short repeat sequence. It is also found among the old-world members, but not in the same location throughout (denoted as a site of differentiation in Table 2). In the corresponding location, the sequence among the old-world members is TTAAGCAATAAAT, however it is not present in all members of the old world.

The previously mentioned 17-base pair deletion occurring among the new-world ciscoes is not apparent in the final alignment (Table 2). This is attributed to the algorithm

program found in MacVector 7.0, which is unable to align the right domain of D-loop with any certainty once many samples consisting of varying lengths, repeats, and in-dels are introduced. Alignment using ClustalW allows for a better comparison over MacVector in this respect, and the deletion is no longer seen. What does become apparent is a significantly larger deletion (PyrPurAPyrPurAAA(T or G)TTTATTAATGTATAATAAAAAGTT) found only among the new-world ciscoes, including *C. autumnalis* pollan. However, this stretch of sequence is found in all of the old-world ciscoes sampled, even occurring twice in a number of them (*C. albula* and one variant each of *C. sardinella*, and *C. peled*). Due to the rather substantial size of the deletion, this would serve as a good genetic marker for the differentiation of old and new-world ciscoes. It could readily be made into a short oligonucleotide probe and used to probe PCR products that amplified the right domain of the D-loop. However, more extensive work in regards to this deletion should be explored before these results can be considered definitive.

Although the aim of the study was to discover a genetic marker capable of discerning old-world from new-world ciscoes, other information regarding the control region of various Coregonines was discernible, as well as relationships among them. While restriction fragment length polymorphism of the mitochondrial DNA of *C. artedi* and *C. hoyi* from the Great Lakes area revealed a 100 base pair insertion present in *C. artedi* and absent in *C. hoyi* (Snyder *et al.*, 1992), no such insertion was noted among the *C. artedi* examined in this study. As previously mentioned, the right domain of the D-loop would be the expected location for such an insertion due to the large amount of variability present in this region. There was one *C. hoyi* sample from Lake Huron that contained an additional 81 base pair replication (data not shown). This is also similar to the tandemly repeated sequences found in the control region of *C. lavaretus* (Brzuzan, 2000).

While only one sequence for Inconnu was obtained, nine other samples were also attempted. Unfortunately, there is a string of guanine bases immediately following the sequencing primer, and the *Taq* polymerase was unable to read through them (data not shown). *Taq* polymerase *in vitro* is without the proof-reading capabilities and the mismatch repair mechanisms found in *in vivo* DNA replication (Hengen, 1995). During long stretches of repeating bases, slippage of the enzyme may occur, resulting in the addition or deletion of bases, or the enzyme "falling off" the template. This results in no product being produced during PCR replication. This problem could be circumvented in the future by employing two sequencing primers, one located further in the phenylalanine tRNA to generate a "running start", and a second primer further downstream.

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Table 1. Fish species represented in this study. N is the number of individuals examined. Fish ID# is the collection number assigned to each individual fish sample upon receipt at the Freshwater Institute. Location of sample indicates the geographic origin of the fish tissue sample.

Nominate taxon	Common name	N	Fish ID#	Location of Sample
C. zenithicus	shortjaw cisco	4	45410, 45412 45411, 45413	Lake Barrow, Alberta, Canada
	shortjaw cisco	3	46298, 46296 46297	George Lake, Manitoba, Canada
	shortjaw cisco	3	45675, 45662 45676	Lake Athapapaskow, Canada
C. artedi	lake cisco	3	45678, 45691 45685	Lake Athapapaskow, Canada
	lake cisco	3	32947, 32949 32948	Lake Nippigon, Ontario, Canada
	lake cisco	4	32709, 32711 32710, 32712	Limestone River, Manitoba, Canada
C. hoyi	bloater	5	33577, 33580 33578, 33581 33579	Lake Michigan, United States
	bloater	5	33572, 33575 33573, 33576 33574	Lake Huron, United States
C. clupeaformis	lake whitefish (Bering race)	3	21880, 21882 21881	Arctic Red River, Northwest Territories, Canada
	lake whitefish (Bering race)	2	22395 22394	Kivalina River, Alaska, United States
	lake whitefish (Bering race)	2	37213 37214	Klutina River, Alaska, United States
C. clupeaformis	lake whitefish (Mississipprace)	3	42914, 42916 42915	Lake Barrow, Alberta, Canada

Table 1 Continued.

Nominate taxon	Common name	N	Fish ID#	Location of Sample
C. laurettae	Bering cisco	1	36805	Kenai River, Alaska, United States
	Bering cisco	5	37501, 37504 37502, 37505 37503	Yukon River, Alaska, United States
C. autumnalis	Arctic cisco	3	28957, 28959 28958	Deadhorse, Alaska, United States
	Arctic cisco	3	32051, 32053 32052	Atkinson Point, Northwest Territories, Canada
	Arctic cisco	4	28550, 28552 28551, 28553	Peel River, Canada
C. autumnalis (pollan)	Arctic cisco	9	36293, 36304 36300, 36305 36301, 36306 36302, 36307 36303,	Lough Neagh, Ireland
C. autumnalis baicalensis	Arctic cisco	4	44395, 44397 44396, 44418	Lake Baikal, Russia
C. albula	vendace	5	36019, 36022 36020, 36028 36021	Lake Inari, Finland
C. albula	vendace	5	71431, 71434 71432, 71435 71433	Lake Spindzius, Lithuania
C. peled	peled	2	44197, 44199	Kotiukan Resevoire, Russia
	peled	3	33820 33823 33825	Lapin River, Russia
C. nasus	broad whitefish	2	44187, 44188	Kotiukan River, Russia

Table 1 Continued.

Nominate taxon	Common name	N	Fish ID#s	Location of Sample
C. sardinella	least cisco	5	37192, 37195 37193, 37196 37194	Arctic Red River, Northwest Territories, Canada
	least cisco	2	42360, 42361	Einiveen River Drainage
	least cisco	2	43296 43294	North Killeak Lake, Alaska, United States
Stenodus leucichthys nelma	Inconnu	1	40803	Buffalo Rapids, Northwest Territories, Canada

Table 2. Alignment of the amplified D-loop region of various coregonids. The region depicted is the –strand in the 5'→3' direction. The * indicate identical bases at that position for all fish. The reference sequence for the alignments, *C. autumnalis* (pollan) species, is indicated in bold, the site of differentiation, and the light strand promoter-like (LSP) sequence are noted. Dashes represent gaps inserted in the sequences to produce an alignment.

C. peled	TTATATTTTA ATCAGGAGTG GCCTTCATGA TAGGGTTGTC GGTGCCAAAG TTTGTTAAGT
C. nasus	ATATATTTTA ATCAGGAGTG GCCTTCATGA TAGGGCTGTC GGTGCCAAAG TTTGTTAAGT
C. autumnalis baicalensis	-TATATTTTA ATCAGGAGTG GTCCTCATGA TAGGGTTGTC GGTGCCAAAG CTTGTTAATT
C. tugun	-TATGTTTTA ATCAGGAGTG GCCTT-ATAA CAGTGTTGTC GGTGCCAAAG TTTGTTAAAT
C. clupeaformis Mississippi race	CGCTATTTTA ATCAGGAGTG GCCTT-ATAA CAGTGTTGTC GGTGCCAAAG TTTGTTAAAT
C. albula	--ATCTTTTA ATCAGGAGTG GCCCGCATGA TGGGGTTGTC GGTGCCAAAG TTTGTTAAGT
C. sardinella	---------- -----GAGTG GCCCGCATGA TGGGGTTGTC GGTGCCAAAG TTTGTTAAGT
C. peled 2	CAATCTTTTA ATCAGGAGTG GCCCTCATGA TAGGGTTGTC GGTGCCAAAG TTTGTTAAGT
C. albula 2	CAATCTTTTA ATCAGGAGTG GCCCTCATGA CAGGGTTGTC GGTGCCAAAG TTT-------
*C. sardinella*2	---TCTTTTA ATCAGGAGTG GCCCTCATGA TAGAGTTGTC GGTGCCAAAG TTTGTTAAGT
C. clupeaformis Bering race	---------A ATCAGAAGTG GCCTTTATGA TAGGGTTGTC GGTGCCAAAG TTTGTTAAGT
***C. autumnalis* (pollan)**	**--ATGTTTTA ATCAGGGGTG GCCCTTATGA CAGGGTTGTA TGTGCCAAAG TTTGTTAAGT**
C. autumnalis	-------TTA ATCAGGGGTG GCCCTTATGA TAGGGTTGTA TGTGCCAAAG TTTGTTAAGT
C. zenithicus	-TATGTTTTA ATCAGGGGTG GCCCTTATGA TAGGGTTGTA TGTGCCAAAG TTTGTTAAGT
C. artedi	-TATGTTTTA ATCAGGGGTG GCCCTTATGA TAGGGTTGTA TGTGCCAAAG TTTGTTAAGT
C. hoyi	-TATGTTTTA ATCAGGGGTG GCCCTTATGA TAGGGTTGTA TGTGCCAAAG TTTGTTAAGT
C. laurettae	--ATGTTTTA ATCAGGGGTG GCCCTTGTGA TAGGGTTGTA GGTGCCAAAG TTTGTTAGGT
S. leucichthys	---CATTCTA ATGGGGGGGG GTCCTTATGA TAGGGTTATC GGTGCCAAAG TTTGTTAAGT
	* * *

Table 2. Continued.

C. peled	ATAACAAAAA TTTTATTAA TGTATACTTT AATTAAGGGG TGGCTTTGAT AATAGGATTGT
C. nasus	ATAACAAAAA TTTTATTAA TGTATACTTT AATTAAGGGG TGGCCTTGAT AACAGGATTGT
C. autumnalis baicalensis	ATAACAAAAA TTTTATTAA TGTATACTTT AATTAAGGGG TGGCCTTGAT AATAGGATTGT
C. tugun	ATAATAAAAA GTTTATTAA TGTATACTTT AATTAA---- ---------- ----------
C. clupeaformis Mississippi race	ATAATAAAAA GTTTATTAA TGTATACTTT AATTAA---- ---------- ----------
C. albula	ATAACAAAAA GTTTATTAA TGTATACTTT AATTAAGGGG TGGCTTTGAT AATAGGATTGT
C. sardinella	ATAACAAAAA GTTTATTAA TGTATACTTT AATTAAGGGG TGGCTTTGAT AATAGGATTGT
C. peled 2	ATAACAAAAA GTTTATTAA TGTATACTTT AATTAAGGGG TGGCCTTGAT AATAGGATTGT
C. albula 2	---------- --------- ---------- ---------- ---------- ----------
*C. sardinella*2	ATAACAAAAA GTTTATTAA TGTATACTTT AATTAAGGGGT GGCTTTGAT AATAGGATTGT
C. clupeaformis Bering race	ATAACAAAAA TTTTATTAA TGTATACTTT AATTAAGGGGT GGTTTTGAT AATAGGATTGT
***C. autumnalis* (pollan)**	**ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTTTTGAT AATAGGATTGT**
C. autumnalis	ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTTTTGAT AATAGGATTGT
C. zenithicus	ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTTTTGAT AATAGGATTGT
C. artedi	ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTTTTGAT AATAGGATTGT
C. hoyi	ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTTTTGAT AATAGGATTGT
C. laurettae	ATAACAAAAA GTTCACTAA TGTATACTTT AATTAAAGGAT GGTCTTGAT AATAGGATTGT
S. leucichthys	ATAACAAAAA GTTCATTAA TGTATACTTT AATTAAAGGGT GGCTTTGAT AATAGGATTGT

LSP

Table 2. Continued.

C. peled	CGGTGCCAAA GTTTGT---- ---------- ---------- ---------- ----------
C. nasus	CGGTGCCAAA GTTTGT---- ---------- ---------- ---------- ----------
C. autumnalis baicalensis	CGGTGCCAAA GCTTGT---- ---------- ---------- ---------- ----------
C. tugun	---------- ---------- ---------- ---------- ---------- ----------
C. clupeaformis Mississippi race	---------- ---------- ---------- ---------- ---------- ----------
C. albula	CGGTGCCAAA GTTTGTTAAG CAATAAAT-- -TTTATTAAT GTATAATAAA AAGTTT--TA
C. sardinella	CGGTGCCAAA GTTTGTTAAG CAATAAAT-- -TTTATTAAT GTATAATAAA AAGTTT--TA
C. peled 2	CGGTGCCAAA GTTTGTTAAG CAATAAAT-- -TTTATTAAT GTATAATAAA AAGTTT--TA
C. albula 2	---------- ----GTTAAG CAATAAAT-- -TTTATTAAT GTATAATAAA AAGTTT--TA
*C. sardinella*2	CGGTGCCAAA GTTT------ ---------- ---------- ---------- ----------
C. clupeaformis Bering race	CGGTGCCAAA GTTT------ ---------- ---------- ---------- ----------
***C. autumnalis* (pollan)**	**CGGTGCCAAA GTTT-TAAGT ATAATAAA-- GTTTATTAAT GTATAATTAA AAGTT--TTA**
C. autumnalis	CGGTGCCAAA GTTT-TAAGT ATAATAAA-- GTTTATTAAT GTATAATTAA AAGTT--TTA
C. zenithicus	CGGTGCCAAA GTCT-TAAGT ATAATAAA-- GTTTATTAAT GTATAATTAA AAGTT--TTA
C. artedi	CGGTGCCAAA GTCT-TAAGT ATAATAAA-- GTTTATTAAT GTATAATTAA AAGTT--TTA
C. hoyi	CGGTGCCAAA GTCT-TAAGT ATAATAAA-- GTTTATTAAT GTATAATTAA AAGTT--TTA
C. laurettae	CGGTGCCAAA GTTT-TAAGT ATAATAAA-- GTTTATTAAT GTATAGTTAA AAGTT--TTA
S. leucichthys	CGGTGCCAAA GTTTGTAAGT ACAATAAAAA GTTTATTAAT GTATACAAAA AGTTTA-TTA

site of differentiation

Table 2. Continued.

C. peled	---------- ---------- ---------- ---------- ---------- ---------A
C. nasus	---------- ---------- ---------- ---------- ---------- ---------A
C. autumnalis baicalensis	---------- ---------- ---------- ---------- ---------- ---------A
C. tugun	---------- ---------- ---------- ---------- ---CGGTGCC AAAGTTTATA
C. clupeaformis Mississippi race	---------- ---------- ---------- ---------- ---CGGTGCC AAAGTTTATA
C. albula	GTGTATACTT TAATTAGGGG GCGGTTTTGA TAATAGGATT GTCGGTGCCA AAGTTTGTTA
C. sardinella	GTGTATACTT TAATTAGGGG GCGGTTTTGA TAATAGGATT GTCGGTGCCA AAGTTTGTTA
C. peled 2	GTGTATACTT TAACTAGGGG GCGGTTTTGA TAATAGGATT GTCGGTGCCA AAGTTTGTTA
C. albula 2	GTGTATACTT TAATTAGGGG GCGGTTTTGA TAATAGGATT GTCGGTGCCA AAGTTTGTTA
*C. sardinella*2	---------- ---------- ---------- ---------- ---------- ------GTTA
C. clupeaformis Bering race	---------- ---------- ---------- ---------- ---------- ------GT-A
***C. autumnalis* (pollan)**	**ATGTATACTT TAATTAAGGG GTGGCTTTGA TAATAGGATT GTCGGTGCCA AAGTTTATTA**
C. autumnalis	ATGTATACTT TAATTAAAGG GTGGCTTTGA TAATAGGATT GTCGGTGCCA AAGTTTATTA
C. zenithicus	ATGTATACTT TAACTAAAGG GTGGCTTTGA TAATAGGATT GTCGGTGCCA AAGTTTATTA
C. artedi	ATGTATACTT TAACTAAAGG GTGGCTTTGA TAATAGGATT GTCGGTGCCA AAGTTTATTA
C. hoyi	ATGTATACTT TAACTAAAGG GTGGCTTTGA TAATAGGATT GTCGGTGCCA AAGTTTATTA
C. laurettae	ACGTATACTT TAATTAAAGG GTAACTCTCA TAATAGGATT GTCGGTGCCA AAGTTTATTA
S. leucichthys	ATGCATACTT -AATTAAAGG GTG-CTTTGA TAATAGGGTT GTCGGTGCCA AAGTTTGT--

Table 2. Continued.

C. peled	AGTAATAAAG TTTATTAATG TATAATAAAA AGTTTTAGTG TATACTTTAA TTAAGGGGTG
C. nasus	AGTAATAAAG TTTATTAATG TATAATAAAA AGTTTTAGTA TATACTTTAA TTAGGGGGTG
C. autumnalis baicalensis	AGTAATAAAG TTTATTAATG TATAATAAAA AGTTTTAGTG TATACTTTAA TTAGGGGGTG
C. tugun	AATAGTAAAT TTTATTAATG TATAATAAAA AGTTTTAGTA TGTACTTTAA TT-GGGGGTG
C. clupeaformis Mississippi race	AATAGTAAAT TTTATTAATG TATAATAAAA AGTTTTAGTA TGTACTTTAA TT-GGGGGTG
C. albula	AGCAATAAAT TTTATTAATG TATAATAAAA AGTTTTAGTG TATATTTTAA TTAGGGGGTG
C. sardinella	AGCAATAAAT TTTATTAATG TATAATAAAA AGTTTTAGTG TATATTTTAA TTAGGGGGTG
C. peled 2	AGCAATAAAT TTTATTAATG TATAATAAAA AGTTTTAGTG TATATTTTAA TTAGGGGGTG
C. albula 2	AGCAATAAAT TTTATTAATG TATAATAAAA AGTTTTAGTG TATATTTTAA TTAGGGGGTG
*C. sardinella*2	AGCAATAAAT TTTATTAATG TATAATAAAA AGTTTTAGTG TATACTTTAA TT-GGGGGCG
C. clupeaformis Bering race	AGTAACAAAG TTTATTAATG TATAATAAAA AGTTTTAGTG TATACTTTAA CTGGGGGGGG
***C. autumnalis* (pollan)**	**A--------- ---------- ---------- ----TTAATG TATATTTTAA TTAAGGGGTG**
C. autumnalis	A--------- ---------- ---------- --------TG TATATTTTAA TTAAGGGGTG
C. zenithicus	A--------- ---------- ---------- ----TTAATG TATATTTTAA TTAAGGGGTG
C. artedi	A--------- ---------- ---------- ----TTAATG TATATTTTAA TTAAGGGGTG
C. hoyi	A--------- ---------- ---------- ----TTAATG TATATTTTAA TTAAGGGGTG
C. laurettae	A--------- ---------- ---------- ----TTAATG TATATTTTAA TTAAGGGGTG
S. leucichthys	-----CAAGT ATAACAAAAG TTTA------ ----TTAATG TATATCTTAA TTAAAGCGTA
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Deletion in New-world ciscoes

Table 2. Continued.

C. peled	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTT---- ---------- --------A-
C. nasus	GCCTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTT---- ---------- --------AA
C. autumnalis baicalensis	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTT---- ---------- --------AA
C. tugun	GCTTTGATAA TAGGGTTGTC GGTGCCAAAG TTTGCTAAGA ATAATAAAAA GCTTGTATAA
C. clupeaformis Mississippi race	GCTTTGATAA TAGGGTTGTC GGTGCCAAAG TTTGCTAAGA ATAATAAAAA GCTTGTATAA
C. albula	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTTGTGT ATA-TAAAAA GTTTAT-TAA
C. sardinella	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTTGTGT ATA-TAAAAA GTTTAT-TAA
C. peled 2	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTGTTGAGT ATA-TAAAAA GTTTAT-TAA
C. albula 2	GCTTTGATAA TAGGGTTGTC GGTGCCAAAG TTTGTTGAGT ATA-TAAAAA GTTTAT-TAA
*C. sardinella*2	GTTTTGATAA TAGGGTTGTC GGTGCCAAAG TTTGTTGAGT ATA-TAAAAA GTTTAT-TAA
C. clupeaformis Bering race	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTT------- ---------- ----AT-TAA
***C. autumnalis* (pollan)**	**GCTTTGATAA TAGGATTGTC AGTGCCAAAG TTTGTTAAGT ATGATAAAAA GTCTAT-TAA**
C. autumnalis	GCTTTGATAA TAGGATTGTC AGTGCCAAAG TTTGTTAAGT ATGATAAAAA GTCTAT-TAA
C. zenithicus	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTATTAAGT ATGATAAAAA GTCTAT-TAA
C. artedi	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTATTAAGT ATGATAAAAA GTCTAT-TAA
C. hoyi	GCTTTGATAA TAGGATTGTC GGTGCCAAAG TTTATTAAGT ATGATAAAAA GTCTAT-TAA
C. laurettae	GCTTTGATAA TAGGATTGTC AGTGCCAAAG TTTGTTAAGT ATAATAAAAA GTCTAT-TAA
S. leucichthys	GTTTTGATAA TAGGGTTGTC GGTGCCAAAG TTTGTTAAGT ATAACAAAA- GTTTAT-TAA
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Table 2. Continued.

C. peled	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. nasus	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. autumnalis baicalensis	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAA
C. tugun	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. clupeaformis Mississippi race	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. albula	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. sardinella	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. peled 2	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. albula 2	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
*C. sardinella*2	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
C. clupeaformis Bering race	TGTATGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTTGGGGT TTGACAGGAA
***C. autumnalis* (pollan)**	**TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAG**
C. autumnalis	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAG
C. zenithicus	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAG
C. artedi	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAG
C. hoyi	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAG
C. laurettae	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGACTTCCTG GTTTCGGGGT TTGACAGGAA
S. leucichthys	TGTACGGTTT GATGGGTAAT AGCGCTATCG AGGCTTCCTG GTTTCGGGGT TTGACAGGAG
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Table 2. Continued.

C. peled	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. nasus	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. autumnalis baicalensis	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. tugun	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. clupeaformis Mississippi race	TAATAAGGAT CGCTCACCGT AGGGGGGTAG GGGGGTTTAG CCGCCCAAAA GCCGGGGAGA
C. albula	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. sardinella	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. peled 2	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. albula 2	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
*C. sardinella*2	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
C. clupeaformis Bering race	TAATAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
***C. autumnalis* (pollan)**	**TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA**
C. autumnalis	TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA
C. zenithicus	TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA
C. artedi	TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA
C. hoyi	TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA
C. laurettae	TAACAAGGAT CGCTCAGCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAGAA GCCGGGGAGA
S. leucichthys	TAATAAGGAT CGCTCACCGT AGGGGGGTAG GGGGGTTTAG CCGCGCAAAA GCCGGGGAGA
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Table 2. Continued.

C. peled	TCTTAGGTAT CTGTGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. nasus	TCTTAGGTAT CTGTGAGGAA AGAAACTGAC CTTATGCACT TGATATAAAT TATGCAATTC
C. autumnalis (baicalensis)	TCTTAGGTAT CTGTGAGGAA GGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. tugun	TCTTAGGTAT CTGTGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. clupeaformis Mississippi race	TCTTAGGTAT CTGTGATGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. albula	TCTTAGGTAT ATATGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. sardinella	TCTTAGGTAT ATATGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. peled 2	TCTTAGGTAT ATATGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAGTTC
C. albula 2	TCTTAGGTAT ATATGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
*C. sardinella*2	TCTTAGGTAT ATCTGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
C. clupeaformis Bering race	TCTTAGGTAT CTGTGAGGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
***C. autumnalis* (pollan)**	**TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC**
C. autumnalis	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC
C. zenithicus	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC
C. artedi	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC
C. hoyi	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC
C. laurettae	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATTAGT TATGCAATTC
S. leucichthys	TCTTAGGTAT CTATGAAGAA AGAAACTGAC CTTATGCACT TGATATCAAT TATGCAATTC
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Table 2. Continued.

C. peled	TTTATAGAAT ATCATTCCGC CATTATTATT GGGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. nasus	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. autumnalis (baicalensis)	TTTATAGAAT ATCATTCCGC CATTATTATT GGGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. tugun	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. clupeaformis Mississippi race	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTATTTCGA
C. albula	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. sardinella	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. peled 2	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. albula 2	TTTATAGAAT ATCATTCCGC CATTAATATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
*C. sardinella*2	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. clupeaformis Bering race	TTTATAGAAT ATCATTCCGC CATTATTATT GGGTCCGCTG GAGACCAAGA TCTAGTTCGA
***C. autumnalis* (pollan)**	**TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA**
C. autumnalis	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. zenithicus	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. artedi	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. hoyi	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
C. laurettae	TTTATAGAAT ATCATTCCGC CATTAATATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
S. leucichthys	TTTATAGAAT ATCATTCCGC CATTATTATT GTGTCCGCTG GAGACCAAGA TCTAGTTCGA
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Table 2. Continued.

C. peled	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. nasus	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. autumnalis (baicalensis)	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. tugun	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. clupeaformis Mississippi race	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. albula	CCCTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. sardinella	CCCTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. peled 2	CCCTGTTAAA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. albula 2	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
*C. sardinella*2	CCCTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. clupeaformis Bering race	CCTTGTTAGA CTTCTGGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
***C. autumnalis* (pollan)**	**CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA**
C. autumnalis	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. zenithicus	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. artedi	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. hoyi	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
C. laurettae	CCCTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAAGCT GAAAGGAAAA AAAAAAAA
S. leucichthys	CCTTGTTAGA CTTCTCGGTG TGCACTTGTA TATGCAACCG GAAAGGAAAA AAAAAAAA
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